Squalene-2,3-Oxide, an Intermediate in the Enzymatic Conversion of Squalene to Lanosterol and Cholesterol*  

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SUMMARY

The conversion of squalene to lanosterol by rat liver preparations in vitro has been re-examined. It has been shown that this transformation, hitherto considered to take place through the agency of one enzyme ("squalene oxidocyclase-1"), actually involves the formation of squalene-2,3-oxide as an intermediate. The oxide is cyclized to lanosterol by a cyclizing system which acts independently of the oxidative step and requires neither oxygen nor NADPH. The cyclase is shown to exist in highest concentration in the microsomal fraction, although an over-all greater quantity of the enzyme remains in the supernatant after centrifugation for 1 hour at 100,000 × g.

Numerous experiments have supported the scheme put forward by Woodward and Bloch for the biological cyclization of squalene to yield lanosterol (1), and the intermediate role of lanosterol in the biosynthesis of cholesterol (2, 3) and ergosterol (4) has been shown. Recent reviews of the relevant literature are available (5, 6). The pattern of folding of the squalene chain proposed by Woodward and Bloch suggested that cyclization was probably initiated by attack of an electrophile (X+) on the double bond at one end of the chain, and this concept has been developed by others (7–9) in schemes which represent the structure of the end-product determined by the conformation of the squalene chain which, in turn, must be the outcome of constraints imposed by the enzyme system that catalyzes the cyclization.

When the cyclization is carried out in the presence of deuterium oxide, no deuterium is found in lanosterol. This result has been interpreted to exclude the formation of partially cyclized intermediates that are stabilized by loss of a proton, since the reactivation of the cyclization of such compounds might be expected to involve the incorporation of a proton from the medium (10).

The rearrangements of methyl groups and hydride ions which must occur in the course of stabilization of the structures of rings C and D of lanosterol has been postulated (8, 10) to take place as a concerted series of 1,2-shifts. The results of several elegant studies which used isotopic labeling methods show that the final outcome of these rearrangements is in complete accord with this hypothesis (11–13).

The exact mechanism of initiation of cyclization has however, remained, until recently, poorly defined. By the use of 18O labeling, Tchen and Bloch (10, 14) showed that the 3β-hydroxyl group of lanosterol was derived from atmospheric oxygen and not from the water of the incubation medium. This result was taken to support the mechanism of initiation of cyclization by OH+ and the view that the enzymatic oxidative and cyclizing activities were probably inseparable was expressed in the naming of the enzyme "squalene oxidocyclase-1." This designation implied that other squalene oxidocyclases must be presumed to be responsible for the conversion of squalene to polycyclic triterpenes other than lanosterol, and in fact the enzymatic conversion of squalene to the pentacyclic triterpene β-amyrin has been shown recently (15). Tchen and Bloch recognized, however, that, on the basis of their results an alternative possibility of proton-initiated cyclization, followed by hydroxylation at C-3, was not entirely excluded, although they reported that attempts to find evidence for it were unsuccessful. Recently some data have been reported which support this possibility (16), but they are unconvincing from the quantitative point of view. The further possibility that enzymatic cyclization of squalene might be initiated by attack of an hydroxyl radical has also been considered (17, 18).

Another possible mechanism, which seemed most attractive to us, involved the initial enzymatic conversion of squalene to its 2,3-oxide (Fig. 1, Route B) and the subsequent initiation of cyclization of this compound by enzymatic electrophilic attack on the oxide ring. Our interest in this possibility arose...
from an extended series of studies of the nonenzymatic, acid catalyzed cyclization of squalene-2,3-oxide and analogous terpenoid oxides whose syntheses are readily achieved by methods developed in one of our laboratories (19). These studies had shown that an electrophilic attack on the oxide ring resulted in fission of the C-2-oxygen bond with cyclization of the carbon skeleton to tricyclic systems. It was noted that the stereochemistry of the products formed in the course of such reactions conformed to that found in rings A and B of the naturally occurring terpenes (Fig. 2) (20-22).

The acid-catalyzed cyclization of analogous oxides has been discussed previously as a formal analogy to the hypothetical OH+-initiated biological cyclization of squalene (23).

If squalene-2,3-oxide is in fact an intermediate in the enzymatic conversion of squalene to lanosterol, it is reasonable to suppose that the step which requires oxygen and NADPH (14) is specifically concerned with the conversion of squalene to its oxide, while lanosterol should be formed from squalene-2,3-oxide anaerobically and without involvement of NADPH. In this paper we describe experiments in which these suppositions have been tested and confirmed. Preliminary accounts of this work have been published (24, 25) and essentially similar results have also been reported in preliminary form by Corey and Russey (26).

**EXPERIMENTAL PROCEDURE**

*Materials—m-Mevalonic acid-5-3H (84.5 mC per mmole) and mL-mevalonic acid-2-3H (5 mC per mmole) were obtained in the form of the dibenzyl ethylenediamine salts from New England Nuclear and were used without further purification. ATP, NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Mann. Squalene was obtained from Eastman-Kodak. The squalene was purified before use by chromatography on silica gel followed by thiourea clathrate formation (27). The product gave only one peak by gas chromatography on a column of polydiethylene glycol succinate. This material was used for all preparative work. Lanosterol was purchased from Aldrich and was found by gas liquid chromatography to consist of a mixture of 60\% lanosterol and 40\% dihydrolanosterol. Pure lanosterol (m.p. 140-141\(^\circ\)) was obtained by precipitation of the acetate dibromide and debronnination and saponification, essentially as described by Johnston, Gantschi, and Bloch (28). Dihydrolanosterol (m.p. 146-148\(^\circ\)) was prepared from purified lanosterol by hydrogenation (29). Cholesterol was obtained from Aldrich and was purified before use by bromination and debronnination as described by Fieser (30).*

*Gas-Liquid Chromatography—Gas-liquid chromatography was carried out by means of a Perkin-Elmer 881 gas chromatograph with all glass columns (6 feet \(\times\) \(\frac{1}{4}\) inch inner diameter) and injection systems, with a flame ionization detector and equipped with an effluent stream splitter which permitted collection from four-fifths of the effluent, while the remaining one-fifth was passed to the detector. Materials corresponding to the emergent peaks were collected by inserting glass capillaries of approximately 1-mm bore through a Teflon gasket attached to the outlet of the gas chromatograph. The efficiency of collection by this technique was 30 to 35\% of the injected sample. Two types of polar liquid phases were used: temperature stabilized polydiethylene glycol succinate obtained as a 5\% coating on silanized Chromosorb W, 100 to 120 mesh (Applied Science Laboratories, Inc., State College, Pennsylvania), and a terminated Carbowax ("Steroid analytical phase," Wilkins Instrument Company, Inc., Walnut Creek, California), which was applied to acid-washed, silanized Chromosorb W at 5\% concentration. Columns of polydiethylene glycol succinate were conditioned at 205\(^\circ\) for 48 hours, then operated routinely at 200\(^\circ\), with inulet and outlet temperatures of 300\(^\circ\) and a nitrogen flow rate of 90 ml per min. Columns of Carbowax were conditioned first at 250\(^\circ\) for 2 hours, then at 230\(^\circ\) for a further 15 hours, then operated at 225\(^\circ\) with a nitrogen flow rate of 120 ml per min and inlet and outlet temperatures of 300\(^\circ\).*

Trimethyl silyl ethers were prepared by treatment of the hydroxyl derivative (<1.0 mg) for 2 hours at room temperature with 0.2 ml of trimethyl silyl chloride and 0.5 ml of anhydrous pyridine. The reagents were then evaporated to dryness under a stream of nitrogen and the products extracted from the residue with hexane. The hexane solution was filtered through a small amount of a mixture of sodium sulfate and anhydrous sodium carbonate, evaporated to dryness and the residue taken up in a few microliters of dry hexane for injection into the gas chromatograph. As a reference compound, cholesterol was added to the injected solution. Retention times are quoted in relation to that of cholestanol as \(R_e\) values.

For the regeneration of free hydroxylic materials from their
trimethyl silyl ethers after recovery from gas-liquid chromatography, the ether was dissolved in a small volume of a mixture of 70 ml of 95% ethanol and 25 ml of 0.1 N aqueous hydrochloric acid. After 1 hour, the solution was diluted with water and the free hydroxyl material was extracted with hexane.

Some analyses were carried out by conversion of the sterols to methyl ethers and separation on polydiethylene glycol succinate as previously described (31).

Radioactivity Measurements—A Packard liquid scintillation counter operating with an efficiency of 41% for $^3$H and 89% for $^14$C was used. Samples were assayed in 20-ml glass vials in the presence of 50 mg of $^3$H- or $^14$C-labeled internal reference was used.

Radiochemical purity of the samples was determined by conversion into squalene-2,3-oxide for which methylene chloride was used alone to avoid the requirement, 25% ethyl acetate in hexane (System II) for the isolation of lanosterol, or 15% ethyl acetate in hexane (System III) for some other preparative purposes. Radioactive incubation products were localized on the plates by chromatographing the bands corresponding to the reference substances were then scraped from the plate and extracted with a suitable solvent. A mixture of methylene chloride and methanol (1:1) was used for extraction of all materials other than squalene oxide for which methylene chloride was used alone to avoid the possibility of hydrolytic attack on the oxide ring. Extractions were carried out by suspending the silica gel in the solvent in a centrifuge tube followed by evaporation of the solvent after centrifugation.

Squalene-2,3-oxide—Squalene-2,3-oxide was prepared by the following procedure, based on the earlier procedure of van Tamelen and Curphey (19).

$N$-Bromosuccinimide, 12.5 g (0.07 mole), was added in small portions over a period of 10 min to a solution of 25 g (0.06 mole) of squalene in 430 ml of tetrahydrofuran and 120 ml of water. The additions were made under an atmosphere of nitrogen which was maintained while the solution was stirred at 5° for a further 60 min. After evaporation of the major portion of the solvent under reduced pressure the residual aqueous mixture was poured into ice water and, extracted with benzene, and the combined benzene extracts were dried over sodium sulfate to yield 29.2 g of crude monobromohydrin. The crude product was dissolved in petroleum ether (b.p. 40–45°) and applied to a column of silica gel (800 g, 15% water) made up in the same solvent. Unchanged squalene and nonhydroxyl bromination products were eluted with petroleum ether. The bromohydrin (11.8 g) was eluted with 20 to 50% benzene in petroleum ether.

The foregoing bromohydrin was dissolved in 250 ml of methanol under nitrogen and 13.8 g of anhydrous potassium carbonate were added in one portion. The mixture was stirred for 1 hour at 25°, evaporated to dryness under reduced pressure, and the residue was extracted exhaustively with ether. The combined ether extracts were washed with water, dried over sodium sulfate, and evaporated under reduced pressure to yield 6.3 g of squalene oxide of better than 95% purity. The purity of small amounts of the oxide was routinely determined by thin layer chromatography in System I in which the oxide moves with an $R_f$ of 0.41 to 0.43.

The following analytical data support the structural assignment.

\[
\text{C}_{30}\text{H}_{50} \text{O} \\
\text{Calculated: C 84.81, H 11.78} \\
\text{Found: C 84.44, H 11.81}
\]

Mass spectrum (cf. Fig. 3a) showed a molecular ion peak at $m/e = 426$, with major fragments of $m/e = 357, 203, 191, 189, 177, 175, 163, 161, 153, 149, 147, 137, 135, 123, 121, 109, and 107$. Infrared spectrum had maxima of 2960, 2920, 2845, 1660, 1450, 1375, and 1245 cm$^{-1}$. Nuclear magnetic resonance spectral data for squalene-2,3-oxide are given in Table I.

<table>
<thead>
<tr>
<th>Assignment</th>
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<tr>
<td>Protons of methyls attached to carbon (C-2) bearing oxygen</td>
</tr>
<tr>
<td>Vinyl methyl + 2H</td>
</tr>
<tr>
<td>Allylic H</td>
</tr>
<tr>
<td>Proton attached to C-3 of oxide ring</td>
</tr>
<tr>
<td>Vinyl H</td>
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### Table I

<table>
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<tr>
<th>Spectrum</th>
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<th>Assignment</th>
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<tr>
<td>ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.18, 1.21</td>
<td>6H</td>
<td>Protons of methyls attached to carbon (C-2) bearing oxygen</td>
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<tr>
<td>1.58, 1.65</td>
<td>20H</td>
<td>Vinyl methyl + 2H</td>
</tr>
<tr>
<td>1.97, 2.03</td>
<td>18H</td>
<td>Allylic H</td>
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<tr>
<td>2.48 (6, $J = 6$)</td>
<td>1H</td>
<td>Proton attached to C-3 of oxide ring</td>
</tr>
<tr>
<td>5.08 (broad)</td>
<td>5H</td>
<td>Vinyl H</td>
</tr>
</tbody>
</table>

* $J$, coupling constant in cycles per sec.
On mass spectrometry, a molecular ion peak was obtained at m/e = 428 with major fragments at m/e = 410, 341, 290, 270, 263, 223, 191, 178, 163, and 149. The infrared spectrum maxima were 3630, 2940, 2865, 1660 (weak), 1524 (weak), 1445, 1375, 1245, and 855 cm⁻¹. Nuclear magnetic resonance spectral data for 2,3-dihydroxy-2-oxosqualene are given in Table II.

Squalene-2,3-glycol and 1,1',2-trisnor-2,3-glycol-3-aldehyde—The preparation of these compounds on a large scale, by methods that are essentially similar to the procedures described (see "Results") for their formation from submilligram quantities of biosynthetically labeled squalene-2,3-oxide, will be described in detail elsewhere.¹ The authenticity of these compounds is based on the following analytical data.

The elemental analysis of squalene-2,3-glycol gave

\[ C_{32}H_{62}O_8 \]

Calculated: C 81.02, H 11.79
Found: C 81.42, H 11.52

On mass spectrometry the molecular ion, m/e = 444, was most abundant, with major fragments of m/e = 429, 426, 385, 375, 367, 357, 290, 231, 149, 143, 137, 123, 109, 95, 81, 69, 59, and 55. The infrared spectrum gave maxima at 3350, 2965, 2929, 2845, 1665, 1450, 1380, and 1060 cm⁻¹. Nuclear magnetic resonance spectral data for 1,1',2-trisnor-2,3-glycol-3-aldehyde are given in Table III.

Table II

<table>
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<tr>
<th>Spectrum</th>
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</tr>
<tr>
<td>Singlet</td>
<td>1.12</td>
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<tr>
<td>Broad singlet</td>
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<td>Vinyl methyl protons</td>
</tr>
<tr>
<td>Multiplet</td>
<td>5.08</td>
<td>5H</td>
</tr>
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</table>

On mass spectrometry the molecular ion, m/e = 384, was most abundant, with major fragments of m/e = 369, 356, 341, 315, 273, 220, 205, 192, 161, 136, 111, 93, 81, 69, and 55. The infrared spectrum was essentially similar to that of squalene with additional bands at 3200 (W) (aldehyde hydrogen stretching) and 1720 cm⁻¹ (carbonyl). Nuclear magnetic resonance spectral data for 1,1',2-trisnor-2,3-aldelyde are given in Table IV.

Preparation of Homogenates of Rat Liver—Homogenates of the livers of male rats (100 to 150 g) were prepared essentially according to the method of Bucher and McGarrahan (32). All operations were carried out at 2-4° and all equipment was precooled in ice. In a typical preparation, the livers of 10 rats were chopped with a razor blade and ground for 5 min, using a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle. The homogenization was carried out in small batches (2 livers per run) with 2.5 ml of buffered medium per gram of tissue. The medium was 0.08 M potassium phosphate buffer, pH 7.4, containing 0.004 M magnesium chloride and 0.03 M nicotinamide. The coarse, unfragmented tissue was filtered out rapidly by pouring the homogenate through surgical gauze, and the filtrates were pooled and kept in ice prior to further processing. For incubations involving "whole homogenate" this filtrate was used as such.

Various subcellular components were obtained by centrifugal fractionation at 2° as follows: (a) "Nuclear fraction," and "Sj" supernatant fraction, obtained by spinning at 10,000 × g for 10 min; (b) "Mitochondrial fraction" and S25 supernatant, obtained by spinning the Sj supernatant fraction at 25,000 × g for 15 min; (c) "Microsomal fraction" and S100 supernatant, obtained by spinning the S25 supernatant at 100,000 × g for 60 min.

The particulate fractions were washed twice by rehomogenization in ice-cold medium, with approximately 0.05 of the original volume of the homogenate, in the case of the nuclear fraction, and once with approximately 0.25 of the original volume in the case of the mitochondrial and microsomal fractions. The washed particulate fractions were recovered by recentrifugation under the same conditions as were used for their first isolation. The washings of the nuclear and mitochondrial fractions were combined with the original supernatants (Sj and Sj, respectively) before proceeding with the next stage of fractionation. The washing from the microsomal fraction was discarded.

The washed particulate fractions were resuspended by brief rehomogenization in a volume of buffer equal to one-half of the total or original volume of homogenate and 5 and 50-µl aliquots of each fraction were taken for protein assay by the method of Lowry et al. (33).

Incubations—Incubations were carried out for 2 hours in a Dubnoff shaking incubator at 37° in stoppered Erlenmeyer flasks containing appropriate volumes of homogenate or subcellular fractions and flushed for 2 min with either oxygen or

Table III

<table>
<thead>
<tr>
<th>Spectrum</th>
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<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td>ppm</td>
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</tr>
<tr>
<td>1.20</td>
<td>8H</td>
<td>Protons of methyls attached to carbon (C-2) bearing oxygen</td>
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<tr>
<td>1.61</td>
<td>22H</td>
<td>Vinyl methyl protons</td>
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<td>1.72</td>
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<tr>
<td>2.05 (multiplet)</td>
<td>6H</td>
<td>Vinyl protons and proton on carbon attached to oxygen</td>
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<tr>
<td>5.19 (multiplet)</td>
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Table IV

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<td>ppm</td>
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<td></td>
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<tr>
<td>1.00 (broad singlet)</td>
<td>6H</td>
<td>Methylene protons</td>
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<tr>
<td>1.08 (singlet)</td>
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<tr>
<td>5.10 (multiplet)</td>
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<td>Vinyl protons</td>
</tr>
<tr>
<td>9.66 (triplet)</td>
<td>1H</td>
<td>Aldehydic proton</td>
</tr>
</tbody>
</table>

¹ E. E. van Tamelen, J. Willett, R. Nadeau, and K. B. Sharpless, unpublished work.
![Diagram](https://example.com/diagram.png)

**Figure 3.** Portions of the mass spectra in the region of the molecular ion peaks, of A, normal squalene-2,3-oxide; B, squalene-2,3-oxide-\(^{18}O\); C, normal lanosterol; and D, lanosterol formed enzymatically from 13.

nitrogen according to the requirements of the experiments. Squalene, squalene-2,3-oxide, and lanosterol were added to the incubation flasks in solution in benzene together with 10 μl of propylene glycol before the addition of other components. On evaporation of the benzene, the added substrate was distributed in a thin film in solution in propylene glycol. Additions of the water-soluble components were made as small aliquots of suitable aqueous solutions immediately before the incubation. When a NADPH-generating system was used, its components were added as follows: NADP, 0.2 ml of a 0.01 M solution; glucose 6-phosphate, 0.5 ml of a 0.01 M solution; and glucose 6-phosphate dehydrogenase, 1 unit in 2 μl of water, per 3 ml of incubation mixture. The incubations were terminated by addition of approximately 2 ml of methanol and 1 ml of 15% aqueous potassium hydroxide per ml of incubation mixture. After thorough mixing, the solution was allowed to stand overnight, then extracted three times with its own volume of hexane. The combined hexane extracts were washed twice with water, dried over sodium sulfate, and evaporated to dryness.

**Squalene-\(^{2}H\)-Squalene-\(^{2}H\)** was obtained by the anaerobic incubation of mevalonic acid-5-\(^{2}H\) (84.5 mC per mmole) with the S8 supernatant of a homogenate of rat liver at a concentration of 0.025 mM (13 μmoles) per 4 ml of homogenate and with the addition of a NADPH-generating system and 1 mg of ATP. The contents of 10 such incubations were combined and saponified, and the nonsaponifiable material was isolated according to the standard procedure. It contained 1.57 × 10⁶ dpm. Purification of the squalene was achieved by thin layer chromatography on alumina, from which the squalene-containing fraction was recovered by elution with hexane, followed by thin layer chromatography in Solvent System I. The recovered squalene fraction (\(R_f = 0.8\)) contained 8.33 × 10⁵ dpm (15.1% yield) in 1.7 mg. It was used as such or diluted with nonradioactive squalene before use. The above preparation is typical of several that have been carried out with both \(^{2}H\)- and \(^{13}C\)-mevalonic acids. The product isolated as described was radiochemically homogeneous on gas-liquid chromatography, on polydiethylene glycol succinate.

**Squalene-2,3-oxide-\(^{18}O\)-Squalene-2,3-oxide-\(^{18}O\)**—This was prepared by a small scale modification of the procedure described for the preparation of unlabeled material.

Biologically labeled squalene-\(^{2}H\) (6.1 mg, 4350 dpm per μg), treated with 2.64 mg of N-bromosuccinimide in a mixture of 0.15 ml of tetrahydrofuran and 0.05 ml of water (30% \(^{18}O\)) and worked up as described above, gave a bromohydrin which was isolated by thin layer chromatography in Solvent System III, with an \(R_f\) of 0.4. Treatment of this material with anhydrous potassium carbonate in methanol and separation of the product by thin layer chromatography in System I gave 1.16 mg of squalene-2,3-oxide-\(^{2}H\)-\(^{18}O\) (\(R_f = 0.41\)), specific activity, 4150 dpm per μg.

The mass spectrum of this material in the region of the molecular ions is shown in Fig. 3b. From measurements of the intensities of the peaks corresponding to \(m/e = 426\) (\(^{10}O\)-oxide, molecular ion) and \(m/e = 428\) (\(^{18}O\)-oxide, molecular ion) it was determined that the product of the above reaction contained \(^{18}O\) in the same abundance (30%) as the water used in the reaction.

**RESULTS**

**Biosynthesis of Squalene-2,3-oxide-\(^{2}H\)—Squalene** (20 μg, 47,100 dpm per μg), biosynthetically labeled from mevalonic acid-5-\(^{2}H\), was incubated with 3 ml of the S8 supernatant of rat liver homogenate in the presence of the NADPH-generating system under an atmosphere of oxygen. Duplicate incubations were carried out with and without the addition of 300 μg of unlabeled squalene oxide. The products were isolated by saponification and extraction with hexane, followed by thin layer chromatography in System I. Squalene-2,3-oxide (\(R_f = 0.41\)), was clearly separated from squalene (\(R_f = 0.8\)), lanosterol (\(R_f = 0.1\)), and cholesterol (\(R_f = 0.045\)). The bands corresponding to squalene, squalene oxide, and total sterols were removed from the plate, extracted, and assayed for radioactivity. Recoveries of 80% of the total initial radioactivity were achieved. The distribution of recovered activity was, in the experiments in which 300 μg of unlabeled squalene oxide were present, as follows: squalene, 69.5%; sterols, 11.1%; squalene oxide, 7.2%. In the incubations from which squalene oxide was omitted, there was a markedly lower incorporation of squalene into the oxide (2.2%) and an enhanced incorporation into the combined sterol fractions (19.3%) while the recovery of unchanged squalene was 68.5%.

**Conversion of Squalene-2,3-oxide-\(^{2}H\) to Squalene-2,3-glycol-\(^{18}O\)**—Squalene-2,3-oxide containing 40,900 dpm that had been isolated from the foregoing incubations in the presence of 300 μg of unlabeled squalene oxide was dissolved in 500 μl of tetrahydrofuran and water (7:3), 250 μl of 3% perchloric acid were added under nitrogen, and the mixture was allowed to stand at 4° for 12 hours. The tetrahydrofuran was evaporated under nitrogen and the product was extracted with six 1-ml portions of cyclohexane. The combined extracts were dried over anhydrous sodium sulfate and concentrated in a stream of nitrogen to yield material with a total of 35,968 dpm.

A sample of this material (31,000 dpm) was subjected to thin layer chromatography in System II, giving a band corresponding...
to the glycol (\(R_F = 0.21\)) from which material having 27,000 dpm was recovered. This represents a conversion of 77\% of the starting material to the glycol.

\textit{Cleavage of Squalene-2,3-\textit{C}}\textsubscript{14} to 1,1',2-Trisnorqualene-3-aldehyde-\textit{H}—A sample of the isolated glycol containing 19,000 dpm was dissolved in 1 ml of tetrahydrofuran. Excess sodium metaperiodate was added, followed by sufficient water to make a homogeneous solution which was allowed to stand at 4\° under nitrogen for 16 hours. The tetrahydrofuran was removed in a stream of nitrogen, and the product extracted in cyclohexane. The extracts were combined, dried, and evaporated in a stream of nitrogen, to yield a crude aldehyde fraction with 15,350 dpm. The bulk of the above product (containing 15,000 dpm) was purified by thin layer chromatography in System I and the band corresponding to the aldehyde (\(R_F = 0.17\)) was eluted. It contained 11,600 dpm (77\%). A portion of the aldehyde (9,400 dpm) was subjected to gas-liquid chromatography on a column of Carbowax. The material showed a single peak with a \(R_F\) of 1.41, identical with that of authentic 1,1',2-trisnorqualene aldehyde, which coincided with the peak of radioactivity. On the assumption of a collection efficiency on gas-liquid chromatography of 30\%, and on the basis of a peak area per \(\mu\text{g}\) relationship obtained with an authentic sample of the aldehyde under the conditions used for this analysis, it was possible to calculate an approximate specific activity of 250 dpm per \(\mu\text{g}\) for the aldehyde. Although the specific activity of the biosynthetic squalene oxide recovered in the presence of squalene oxide added as a carrier was not ascertained, it can be calculated, assuming no conversion of the unlabeled material (300 \(\mu\text{g}\)), that it would be 225 dpm per \(\mu\text{g}\). Since metabolism of the unlabeled oxide under the conditions of these experiments would not be expected to exceed about 10\% (see below), the specific activity of the aldehyde derivative is in excellent agreement with this value.

\textit{Conversion of Squalene-2,3-oxide to 2,3-Dihydro-2-hydroxy Squalene—}From another experiment in which 1400 \(\mu\text{g}\) of squalene-2,3-oxide (4350 dpm per \(\mu\text{g}\)) was incubated under oxygen with a total of 20 ml of the \(S_1\) fraction, the nonvolatile material was isolated in the usual way and subjected to thin layer chromatography in System I. The band corresponding to squalene oxide (\(R_F = 0.33\)) yielded 1 mg of material that had 135,000 dpm. Two further purifications by thin layer chromatography in the same system gave material of the same \(R_F\) which now contained 27,000 dpm. It was dissolved in dry, redistilled tetrahydrofuran and treated with excess lithium aluminum hydride. By extraction of the acidified reaction mixture with ether in the usual manner, a product was obtained, which contained 26,800 dpm. This was subjected to the usual procedure for the preparation of trimethyl silyl ethers and the product was analyzed by gas liquid chromatography on polyethylene glycol succinate. The effluent materials were collected and assayed for radioactivity at 1-min intervals. The only radioactive peak to be detected corresponded in retention time (\(R_F = 1.28\)) with that of authentic 2,3-dihydro-2-hydroxy-squalene trimethyl silyl ether. From a calibration of the response of the gas chromatograph under the conditions of the experiment and assuming 30\% collection efficiency, it was possible to calculate an approximate specific activity for the radioactive alcohol of 4300 dpm per \(\mu\text{g}\).

\textit{Enzymatic Conversion of Squalene-2,3-oxide to Lanosterol and 24,25-Dihydrolanosterol—}Squalene-2,3-oxide-\textit{H} (600 \(\mu\text{g}\), 650 dpm per \(\mu\text{g}\)) was incubated anaerobically with six 3-ml aliquots of the \(S_1\) supernatant fraction of rat liver containing the NADPH generating system. The nonvolatile fraction (300,000 dpm) was isolated in the usual way and subjected to thin layer chromatography on a plate (20 \(\times\) 20 cm) in Solvent System II. The band corresponding to lanosterol (\(R_F = 0.44\)) was eluted, yielding a material that contained 35,000 dpm.

A portion of this material (33,000 dpm) was converted to the trimethyl silyl ethers and analyzed by gas-liquid chromatography on a column of Carbowax with efficient collection during 2-min intervals. The collected fractions were assayed for radioactivity and only two radioactive peaks were detected. Peak I (\(R_F = 2.7\)) corresponded to 24,25-dihydrolanosterol trimethyl silyl ether and yielded 7180 dpm of \(^{14}\text{C}\); Peak II (\(R_F = 3.7\)) corresponded to lanosterol trimethyl silyl ether and yielded 3850 dpm of \(^{14}\text{C}\). There was negligible radioactivity in other parts of the chromatogram. In four subsequent experiments the ratios of the areas and associated radioactivity of the lanosterol and dihydrolanosterol peaks varied from approximately 2:1 to 1:2 as in the present experiment.

\textit{Further Identification of Materials of Peaks I and II—}From a similar experiment to the above, in which squalene-2,3-oxide-\textit{H} (6000 dpm per \(\mu\text{g}\)) was incubated with the \(S_1\) supernatant containing a NADPH-generating system, materials corresponding to Peaks I and II were isolated.

Material corresponding to Peak I (17,350 dpm) was combined and crystallized with 20 mg of unlabeled dihydrolanosterol giving 18 mg of material with 800 dpm per mg, which was unchanged during four recrystallizations from methanol and methylene chloride. Acetylation at room temperature with acetic anhydride and pyridine, followed by four further crystallizations from acetone, gave dihydrolanosterol acetate, m.p. 120–121\°, the specific activity of which remained constant at 730 dpm per mg.

A sample of the material of Peak II which contained 35,000 dpm was dissolved in 5% acetic acid in ethyl acetate and hydrogenated at 25\° and at atmospheric pressure in the presence of a platinum catalyst. The product was isolated by evaporation of the solvent, converted to the trimethyl silyl ether, and subjected to gas-liquid chromatography with collection of effluent material. Only one radioactive peak emerged, containing 14,180 dpm. Its retention time (\(R_F = 2.7\)) corresponded with that of 24,25-dihydrolanosterol trimethyl silyl ether.

\textit{Identification of Lanosterol as Lanosteryl Acetate 24,25-Dibromide—}The foregoing results are consistent with the enzymatic conversion of squalene-2,3-oxide to a mixture of lanosterol and dihydrolanosterol. Further evidence for the composition of this mixture was obtained as follows. A portion of material, isolated by thin layer chromatography as described above and containing 14,100 dpm \(^{14}\text{C}\), was combined with 2 mg of pure lanosterol. It was acetylated by overnight treatment with pyridine and acetic anhydride followed by removal of the excess reagents under a stream of nitrogen. Thin layer chromatography of the product in System II gave 1.72 mg of a white solid, \(R_F = 0.58\) (corresponding to lanosteryl acetate) containing 11,000 dpm of \(^{14}\text{C}\). This product was diluted to 25 mg with unlabeled lanosteryl acetate (m.p. 130–131\°) and crystallized to give 21 mg of material with a specific activity of 431 dpm per mg, which remained unchanged during four crystallizations. A portion of the recombined, recrystallized material and mother liquors (19.6 mg) was dissolved in 0.8 ml of acetic acid saturated with potassium.
bromide and treated with 25 μl of 10% bromine in acetic acid (0.045 mmole). After 10 min at room temperature, 30 μl of water were added and the mixture was allowed to stand at 4° overnight. The crystalline dibromide was collected by centrifugation and washed with cold acetic acid, followed by two portions of cold methanol. The crystals were dried in a stream of nitrogen to yield 10.93 mg of white crystalline material (specific activity, 276 dpm per mg). On recrystallization from acetone, 6.10 mg of pure crystalline dibromide, m.p. 167–169° were obtained, with a specific activity of 91 dpm per mg, which was unchanged (91 to 96 dpm) on two further crystallizations. These results are consistent with the presence of 28 to 30% of the radioactivity in lanosterol.

Enzymatic Conversion of Material of Peak II (Lanosterol) to Cholesterol—Material corresponding to Peak II, having 4600 dpm 14C, was distributed between two 3-ml aliquots of S1 supernatant which contained the NADPH-generating system and incubated for 2 hours under oxygen. The nonsaponifiable fraction was isolated in the usual way and subjected to thin layer chromatography in Solvent System II. Of the recovered radioactivity, 1100 dpm (32%) were associated with the cholesterol fraction; 1100 dpm remained associated with lanosterol, and a further 15% was recovered from an intermediate fraction. The radioactive material moving chromatographically with cholesterol and containing 1000 dpm was combined with 20 mg of cholesterol and crystallized to give 18 mg of material having a specific activity of 50 dpm per mg. This material was acetylated with acetic anhydride and pyridine and the product was crystallized from 50% dpm per mg. This material was acetylated with acetic anhydride and pyridine and the product was crystallized from methanol to give 15 mg of cholesterol acetate, m.p. 116°, with a specific activity of 45 dpm per mg, which was unchanged on a second crystallization. A portion of this material, 10 mg, on precipitation as the dibromide and debromination by treatment with zinc dust, gave on crystallization from methanol and methylene chloride 6 mg of cholesterol acetate having 40 dpm per mg, which was unchanged on two further crystallizations.

Enzymatic Conversion of Squalene-2,3-oxide to Cholesterol under Aerobic Conditions—Squalene-2,3-oxide-14C (100 μg, 1100 dpm per μg) was incubated under oxygen with 3 ml of the S1 supernatant containing a NADPH-generating system. The reaction was terminated after 2 hours and the nonsaponifiable fraction (8.2 × 108 dpm) was isolated in the usual way and analyzed by thin layer chromatography in Solvent System II. The material moving with an Rf of 0.28, corresponding to cholesterol, contained 35,000 dpm; 30,000 dpm were recovered from the band corresponding to squalene-2,3-oxide (Rf = 0.80) and material having 5000 dpm was recovered from the area corresponding to lanosterol (Rf = 0.44). A portion of the radioactive material with the mobility of cholesterol on thin layer chromatography, which contained 20,000 dpm, was combined with 20 mg of cholesterol and recrystallized from methanol with no loss of specific activity. The material was acetylated, and the acetate was purified by conversion to the dibromide and debromination with zinc (26) to yield, after crystallization from methanol, 12 mg of material, m.p. 148–149°, specific activity 800 dpm per mg (theory, 900 dpm per mg).

A sample of the remaining material isolated by thin layer chromatography, which contained 5000 dpm, was analyzed by gas-liquid chromatography of the methyl ether on polydiethylene glycol succinate, giving a major peak with Rf = 3.85 corresponding to cholesterol, from which 1500 dpm were recovered. Smaller amounts of activity totaling 100 dpm were associated with the area of the chromatogram corresponding to Δ7 cholesterol methyl ether (Rf = 4.7). When a sample (200 μg) of the labeled cholesteryl acetate, which had been purified by the dibromide, was converted to the methyl ether and analyzed by gas-liquid chromatography under similar conditions, all recovered radioactivity was associated with the cholesteryl methyl ether peak (Rf = 3.85). In this case the effluent materials corresponding to the peak were collected as three consecutive fractions which showed relative specific activities of 5.2, 5.0, and 5.3 dpm per cm², respectively.

In a similar experiment reported previously (24), no loss of specific activity was detectable during purification of the cholesteryl acetate as the dibromide, and when the material was converted to the methyl ether and analyzed by gas-liquid chromatography on polydiethylene succinate, the peak corresponding to cholesteryl methyl ether contained all of the radioactivity that was associated with cholesterol on thin layer chromatography.

Distribution of 2,3-Oxidosqualene Cyclizing Activity in Subcellular Fractions of Homogenate—To determine the intracellular localization of the cyclizing enzyme system, the various fractions of the homogenate, prepared as described, were tested, both individually and in combination with the supernatant, for their capacity to convert squalene-2,3-oxide-14H to lanosterol under anaerobic conditions. A comparison was also made of the activity of the enzyme system in the presence and absence of the NADPH-generating system.

Each 3 ml of incubation mixture were incubated with 30 μg of squalene-2,3-oxide-14H, 5000 dpm per μg. The nonsaponifiable fraction was isolated in the usual way and separated by thin layer chromatography in System II. The bands corresponding to squalene-2,3-oxide and lanosterol (the only significantly labeled areas) were assayed for radioactivity, and the conversion of squalene-2,3-oxide to lanosterol was calculated from the amount of radioactivity associated with the lanosterol band, as a percentage of total recovered nonsaponifiable material. Control incubations carried out with boiled enzyme preparations showed that the amount of activity of squalene-2,3-oxide which appeared in the lanosterol region on thin layer chromatography as a result of nonenzymatic transformation was less than 5% of the lowest enzymatic conversion, and therefore no correction was applied. The protein contents of individual fractions were assayed, and the results are reported in Table V as specific activities (micrograms of lanosterol formed per mg of protein) and the percentage of total recovered cyclizing activity per fraction.

Enzymatic Conversion of Squalene-2,3-oxide-14H,18O to Lanosterol-3H,18O—A sample (750 μg) of squalene-2,3-oxide-14H,18O, 4,150 dpm per μg, 30% 18O, was incubated anaerobically for 2 hours with a total of 60 ml of washed microsomal fractions without addition of the NADPH-generating system. Isolation of the nonsaponifiable fraction in the usual way gave 7.62 mg of material containing 2.1 × 105 dpm. Thin layer chromatography of this product in Solvent System II gave a band with Rf = 0.43, corresponding to lanosterol, which was recovered from silica gel and contained 240,000 dpm. A second separation by thin layer chromatography in the same solvent system gave approximately 100 μg of material, which contained 200,000 dpm and was converted to its trimethyl silyl ether by the standard procedure.

This derivative was now analyzed by gas-liquid chromatography on polydiethylene glycol succinate under standard conditions in four consecutive runs in which the effluent material
TABLE V
Distribution of 2,3-oxido-squalene cyclase activity in fractions of rat liver homogenates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Oxide cyclizeda µg/mg protein</th>
<th>Distribution of recovered cyclizing activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.30</td>
<td>(100)</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>0.15</td>
<td>9.4</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>0.30</td>
<td>9.4</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>1.10</td>
<td>37.5</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.30</td>
<td>44.0</td>
</tr>
<tr>
<td>Nuclei plus supernatantb</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Mitochondria plus supernatantc</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Microsomes plus supernatantd</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Microsomal fraction plus NADPH-generating system</td>
<td>1.10</td>
<td></td>
</tr>
</tbody>
</table>

a For conditions see "Experimental Procedure." Average of two experiments in duplicate.

b Values for individual fractions calculated as the percentage of total recovery. Total recovery of activity in homogenate fractions was actually 105 to 110% of activity in the unfractionated homogenate. The significance of this result is not clear at the present time.

c Each fraction (1.5 ml) was prepared as under "Experimental Procedure."

d was collected. The products recovered from one run with collection of fractions during 1-min intervals were assayed for radioactivity. Two peaks of material emerged. One (Rc = 1.6) contained no radioactivity, while the other (Rc = 3.7, identical with lanosterol trimethyl silyl ether) was associated with all of the recovered radioactivity. The combined recovered materials corresponding to this peak obtained from the following three runs were analyzed by mass spectrometry. The mass spectrum is shown in Fig. 2d and may be compared with that of normal lanosterol (Fig. 2c). Calculation of the abundance of 18O from the relative intensities of the peaks corresponding to the molecular ions with m/e = 498 (18O-lanosterol-trimethyl silyl ether) and m/e = 500 (18O-lanosterol-trimethyl silyl ether) shows the presence of 29% 18O in the recovered material.

DISCUSSION

The experiments described in this paper were designed to explore the possibility that the conversion of squalene to lanosterol, hitherto considered to take place as the result of the activity of a single enzyme, "squalene oxidocyclase-1" (14) according to Route A (Fig. 1), actually entailed at least two steps. These are depicted as Route B (Fig. 1) and involved (a) the oxidation of squalene to its 2,3-oxide and (b) the cyclization of this compound by an acid-initiated process to give lanosterol. It is implicit in the proposed mechanism of the cyclization step that the oxygen of the 2,3-oxide ring should become incorporated into the lanosterol molecule in the form of the 3β-hydroxy group. All of the results reported in this paper are in accord with the sequential oxidation and cyclization mechanism.

When an homogenate of rat liver was incubated with radioactive squalene and an exogenous pool of unlabeled squalene-2,3-oxide, radioactivity accumulated in squalene-2,3-oxide and the incorporation of radioactivity into the sterols was correspondingly reduced, in comparison with control incubations to which no unlabeled oxide was added. The conclusion that the accumulated radioactivity resides in squalene-2,3-oxide is based on the following observations. The radioactivity moves with authentic squalene oxide on thin layer chromatography in 5% ethyl acetate in hexane, a solvent system which clearly separates squalene-2,3-oxide from squalene as well as from isomeric oxides of squalene and squalene-2,3- and 22,23-dioxide. When the labeled 2,3-oxide was hydrolyzed under aqueous acidic conditions, a single product was obtained, which had the mobility on thin layer chromatography of 2,3-dihydro-2,3-dihydroxy squalene and showed no loss of radioactivity. When this material was oxidized with periodate, a new radioactive derivative was obtained which had the mobility on both thin layer chromatography and gas-liquid chromatography of authentic 1,1',2-trimethylsqualene aldehyde and had a specific activity close to that of the oxide. On reduction with lithium aluminum hydride, the labeled 2,3-oxide gave a single product which both on thin layer and gas-liquid chromatography (as the trimethyl silyl ether) had the mobility of 2,3-dihydro-2,6-dihydroxy squalene and retained the original specific activity.

The various derivatives utilized in the above characterization procedures have been synthesized independently in our laboratory and fully characterized. All analytical data available are consistent with the postulated structures, and the identity of the biologically labeled material with squalene-2,3-oxide is strongly supported by the retention of labeling through the reactions described. Its conversion to cholesterol under aerobic incubation conditions gives further evidence for the role of squalene-2,3-oxide as a normal intermediate in the enzymatic conversion of squalene to sterols.

The first 3β-hydroxy sterol to arise from the cyclization of squalene-2,3-oxide, according to Route B, Fig. 1, would be expected to be lanosterol; but, on anaerobic incubation in the presence of a NADPH-generating system and the 81 supernatant of the homogenate, there should also be an appreciable conversion of lanosterol to dihydrolanosterol, mediated by a microsomal NADPH-dependent Δ8-reductase (34). Lanosterol and dihydrolanosterol were identified as products of the incubation of 18O-labeled squalene oxide with the 81 supernatant, first on the basis of their mobilities on thin layer chromatography as the free sterols and the acetates, and on gas-liquid chromatography as the trimethyl silyl ethers. The radioactive material associated with the peak of lanosterol trimethyl silyl ether, on gas-liquid chromatography, was further identified by hydrogenation to a product which, as the trimethyl silyl ether, had a retention time identical with that of dihydrolanosterol. Radioactive material isolated from the original incubation mixture and having the retention time of dihydrolanosterol on gas-liquid chromatography (as the trimethyl silyl ether) showed no loss of radioactivity when co-crystallized with authentic dihydrolanosterol either as the free sterol or after acetylation.

Furthermore, when a portion of the labeled material which behaved like lanosterol on thin layer chromatography was combined with pure lanosterol, acetylated, and recrystallized, no loss of radioactivity was detected. When this mixture was brominated, however, the resulting lanosterol-3β-acetate-24,25-dibromide lost more than two-thirds of its radioactivity. These
results are consistent with the presence of the radioactivity of the material isolated by thin layer chromatography, in a mixture of lanosterol and dihydroxilane-sterol, since it is well known that the acetates of these substances are extremely difficult to separate by crystallization (35), but are readily separable on the basis of the reactivity of the Δ5-bond to bromine (28). The further metabolism of the lanosterol in the experiments to cholesterol under anaerobic conditions gives additional support for authenticity of the cyclization product.

A direct demonstration of the metabolic conversion of squalene-2,3-oxide to lanosterol as the sole product, under anaerobic conditions, was achieved in the experiment involving the anaerobic incubation of squalene-2,3-oxide-18O-3H with washed microsomes. A yield of lanosterol of approximately 7% was obtained from 18O in the course of the lanosterol by gas-liquid chromatography of the trimethyl acetates of these substances are extremely difficult to separate of the reactivity of the AX-bond to bromine (28). The further conversion of squalene to lanosterol is of the reactivity of the AX-bond to bromine (28).

The quantitative retention of 18O in the course of the preparation of 2,3-oxidosqualene cyclase from hog liver has been described (37).

It is clear that the greatest concentration of cyclase activity per mg of protein is in the microsomal fraction. Calculation of the over-all distribution of total cyclizing activity, however, indicates that a large portion of it (though at a lower specific activity) is present in the cell sap. It seems probable that, at least in part, this reflects a failure of some small microsomal fragments to sediment under our conditions of centrifugation. The alternative possibilities, that some degree of solubilization of the enzyme occurs during homogenization or that there is a portion of the cyclizing activity which is normally present in a soluble state, encourages us to believe that true solubilization of the enzyme may be accomplished.4

It is evident from the results shown in Table V that the cyclizing enzyme system is equally effective with or without the addition of the NADPH-generating system. The lack of a requirement both for NADPH and for oxygen is consistent with the concept that the cyclization is an acid-initiated process that does not involve either oxidative or reductive steps. The results are in accord with the expectation that the dependence on these cofactors, originally attributed to the “squalene oxidocyclase” system (14), must actually reflect the requirements of the oxidase system. The characteristics of this enzyme system have not so far been established in our laboratory, but are under examination at the present time.

The results reported here strongly support the proposed role of squalene-2,3-oxide as a naturally occurring intermediate in the biological conversion of squalene to lanosterol. It seems highly probable that the oxide plays a similar role in the biogenesis of the extensive array of tetra- and pentacyclic triterpenes hitherto considered as cyclization products of squalene. Some aspects of the analogy of these biological cyclizations to nonenzymatic cyclizations of squalene-2,3-oxide have been noted elsewhere (37).

It is clear that the identification of the 2,3-oxide of squalene as an intermediate in the cyclization of squalene allows a wide range of new approaches to the problem of the mechanism of cyclization. Not only is the enzyme that catalyzes the cyclization a simpler and more tractable entity than “squalene oxidocyclase-1” was believed to be, but the possibility of dealing with a substrate (the oxide) which is asymmetrical, rather than with squalene itself, which is symmetrical, facilitates the synthesis of a variety of analogues which can be used to test specific concepts concerning the mechanism of action of the cyclase. Current studies in our laboratories are being directed along these lines.

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